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AMPEROMETRIC BIOSENSORS BASED ON 3-DIMENSIONAL HYDROGEL-FORMING EPOXY NETWORKS

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1. INTRODUCTION

Proteins and glycoproteins of enzymes behave with respect to electron transport, but not ion transport, as good electrical insulators. We have shown that these insulators can be made sufficiently electron conducting to allow the flow of a current between reaction centers of redox enzymes and electrodes equaling or exceeding the current associated with the turnover of the enzymes. In order for such a current to flow it is necessary to introduce into the enzyme proteins or glycoproteins fast electron relaying centers, so as to reduce the electron transfer distances.[1,2]

2. METHODS OF INTRODUCING FAST ELECTRON RELAYS INTO ENZYMES

There are three ways by which fast electron relays can be introduced into an enzyme: (a) Covalent bonding of relays to the enzyme's protein;[3-5] (b) covalent bonding through a sufficiently long and flexible tether to a peripheral oligosaccharide of a glycoprotein enzyme;[6] (c) forming complexes between enzyme proteins and redox macromolecules.[1,7]

2.1. COVALENT ATTACHMENT OF ELECTRON RELAYS TO ENZYME PROTEINS.

Relays can be attached to lysine-amines of enzyme proteins through carbodiimide coupling with carboxylic acid derivatives of relays.[3,4] The resulting enzymes can be directly and continuously electrooxidized if reduced

by their substrates. The relay-modified enzymes retain most of their activity and selectivity. For example, glucose oxidase can be chemically modified by covalent attachment of 13±2 electron relaying ferrocenes to its protein. The $FADH_2$ centers of the modified enzyme are electrooxidizable in the absence of diffusional mediators.

2.2. COVALENT ATTACHMENT OF TETHERED ELECTRON RELAYS TO OLIGOSACCHARIDES ON THE PERIPHERY OF ENZYMES.

Periodate oxidation of surface oligosaccharides on enzymes produces aldehydes. These form Schiff bases with electron-relays that are attached through tethers to terminal amines. The Schiff bases can then be reduced with sodium borohydride to form hydrolytically stable secondary amines. Investigation of tethered ferrocenes as relays shows that efficient electron relaying requires sufficiently long tethers for the relay to reach the midpoint between the reactive center of the redox enzyme and its periphery. When the tethers are too short electron relaying is inefficient; and after a certain length, further lengthening of the tether does not improve the electron-relaying efficiency. For glucose oxidase the required tether has 11-13 carbon atoms or carbon and nitrogen atoms.[6]

2.3. ELECTRON RELAYING THROUGH WATER SOLUBLE REDOX MACROMOLECULES: ELECTRON TRANSPORT IN ENZYME: REDOX POLYMER CONJUGATES.

Interaction between water soluble biomolecules is common in nature, forming the basis for immune reactions, DNA replication and recognition systems. Water-dissolved enzyme molecules can be designed to couple to appropriately designed water soluble fast redox macromolecules. The binding may involve hydrophobic, ionic or hydrogen bonding interactions. The redox molecule and the enzyme protein or glycoprotein interpenetrate in the coupling reaction. As a result, redox centers of the enzymes are brought into electrical contact with those of the electron relaying redox macromolecules.[1,7]

The enzyme-complexing redox macromolecules are polyelectrolytes with fast, nitrogen-complexed $[Os(bpy)_2\ Cl]^{+/2+}$ redox centers. The backbones can be based on poly(vinyl pyridine) or other polymers having $Os^{2+/3+}$ complexing functions. An example of such a macromolecule is shown in Figure 1.

Figure 1: Example of an electron relaying, enzyme-complexing redox macromolecule.

2.4. SIMPLE BIOSENSORS BASED ON COMPLEXES OF REDOX ENZYMES AND REDOX MACROMOLECULES.

When an enzyme binding redox macromolecule is adsorbed on or attached to an electrode surface, it will complex dissolved enzymes and electrically connect these.[7] Thus amperometric biosensors can be made in a

process involving two adsorption and rinsing steps. Such biosensors have risetime shorter than 1 second.

A simple biosensor can be made by adsorbing a polymer similar to that shown in Figure 1, with $R_2 = CH_3$, on a carbon electrode, rinsing and complexing the enzyme to transiently non-adsorbed segments of the electrode adsorbed redox macromolecule. For such complexing, the redox macromolecule must be long, because a sufficient number of adsorbed segments is needed in order to avoid desorption; yet most of its segments must be "in solution", i.e. transiently desorbed, so as to complex and penetrate the enzyme protein.[1]

3. CROSSLINKED, 3-DIMENSIONAL ELECTRON-TRANSPORTING ENZYME-REDOX POLYMER NETWORKS: ELECTRICAL "WIRING" OF ENZYMES.

The enzyme-redox polymer adducts of Section 2.4 can be crosslinked if R_2 of Figure 1 is an amine, e.g. CH_2-CH_2-NH_2, or a carboxylic acid.

In the first case, the amines of the redox polymer and of the enzyme can be crosslinked with a bi-functional crosslinker, such as a diepoxide. In the second case the carboxylic acids can be esterified to form reactive N-hydroxysuccinimides. The latter react with lysine amines of the enzyme, the enzyme acting as a crosslinker of the redox macromolecule.[8]

3.1. ENZYME-WIRING REDOX EPOXY HYDROGEL NETWORKS.

The redox polyamines of section 3 can be crosslinked with a water soluble diepoxide, such as polyethylene glycol diglycidyl ether, that does not complex either the enzyme or the redox macromolecule.[9,10] This is important, because complexing of either component can lead to the breakup of the complex in which the enzyme is "wired". The polyethylene glycol diglycidyl ethers used are of 400-600 daltons. Their chains are flexible and well hydrated. With both the enzyme and the redox polymer being water soluble, a hydrogel is formed upon crosslinking. This hydrogel is not only permeable to the substrate and the product of the enzyme catalyzed redox reaction but also has an electron-transporting polymer scaffold.

Consequently, the substrate and product diffuse easily to and from the network-bound enzyme, while the network electrically connects the redox centers of the bound enzymes to electrodes. The current densities and sensitivities of the resulting biosensors are high: current densities in excess of 10^{-3}A cm^{-2} are observed and sensitivities reach 0.1A cm^{-2} M^{-1}.

3.2. MINIATURIZATION OF AMPEROMETRIC BIOSENSORS.

The enzymes in the redox epoxy hydrogels are well "wired", i.e. the electrons associated with their turnover are efficiently collected at the electrodes. In Faraday cages we have observed the turnover of as few as 300 enzyme molecules.

In practical applications biosensors must, however, function in an environment with electromagnetic noise. The noise equivalent currents in our laboratory are near 10^{-11}A. Thus, unless the biosensors are operated in Faraday cages, the currents must exceed 10^{-10}A. For this reason our practical microsensor tips are miniaturized to 7μm diameter, but not less. Electron transport to these tips is radial and current densities in excess of 3mA cm^{-2} are observed at high substrate (e.g. glucose) concentrations.[11]

4. ELIMINATION OF INTERFERANTS.

Biosensors are only imperfectly selective, because non-enzyme-catalyzed electrode reactions may also take place. Thus, on anodes poised positive of the redox potential of the enzyme-wiring redox macromolecule biological fluid constituents such as urate, ascorbate, and acetaminophen may also be electrooxidized. Their electrooxidation currents add to and distort the true, substrate-associated current.

To eliminate the electrooxidizable interferants we use the very fact that they are easy to oxidize. Specifically the interferants are rapidly oxidized by hydrogen peroxide in the presence of horseradish peroxidase. Thus ascorbate, urate, acetaminophen and other interferants are quantitatively eliminated in immobilized horseradish peroxidase overlayers on enzyme electrodes that are electrically insulated from the sensing layer. The fluid reaching the sensing

layer is stripped of all interferants, while species detected such as lactate or glucose are not oxidized.[12]

5. MINIATURE FLEXIBLE PHYSIOLOGICAL LACTATE SENSOR.

The concepts of 3-dimensional wired enzyme hydrogels and interference elimination have been implemented in biosensors of which a miniature physiological lactate sensor is an example. The sensor is 0.3mm in diameter, consisting of 300-500 carbon fiber tips. The fibers are epoxy-embedded and contained in a 0.3mm diameter biocompatible polyimide tubing. The ensemble of epoxy-embedded fiber tips is coated with the redox epoxy hydrogel containing lactate oxidase. This sensing layer is then coated with an electrically insulting layer made of poly(vinyl-imidazole), crosslinked with ethylene glycol diglycidyl ether. The top overlayer contains two coimmobilized enzymes: glucose oxidase and horseradish peroxidase. (Figure 2)

Figure 2: Schematic drawing of the 0.3 mm diameter flexible physiological lactate sensor.

The physiological fluid, e.g. blood, contains glucose and oxygen. Glucose is oxidized by oxygen in the glucose oxidase and horseradish peroxidase containing overlayer in a reaction generating gluconolactone and hydrogen peroxide. The hydrogen peroxide generated oxidizes the horseradish peroxidase, that, in turn, oxidizes and eliminates all the electrooxidizable interferants, but not lactate. The sensing layer sees, therefore, a preoxidized stream from which the interferants are stripped. The electrode is then overcoated with a biocompatible film. The electrode's sensitivity is 2.5×10^{-2} A $cm^{-2} M^{-1}$. It can be stored at $4^{o}C$ for 4 months with no measurable change and its 10-90%. Its risetime is 1 min. If periodically recalibrated, the electrode can be used for 10^{3} lactate measurements.

Acknowledgements:

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Captions:

Figure 1: Example of an electron relaying, enzyme-complexing redox macromolecule.

Figure 2: Schematic drawing of the 0.3 mm diameter flexible physiological lactate sensor.

Biography:

Adam Heller received his M.Sc. (Chemistry and Physics, 1957) and Ph.D. (Chemistry, 1961) from the Hebrew University, Jerusalem, where he studied under E. D. Bergmann. Following postdoctoral work at U.C. Berkeley (with A. Streitweiser) and at Bell Laboratories (with E. Wasserman), he joined GTE Laboratories where he became Manager of Exploratory Research in 1970. In 1975 he returned to ATT Bell Laboratories, heading from 1977 until 1988 the Electronic Materials Research Department. He was appointed to the Ernest Cockrell, Sr. Chair in Engineering at the University of Texas at Austin in 1988.

Dr. Heller is best known for pioneering the direct electrical connection of redox centers of enzymes to electrodes, and the associated direct amperometric biosensors (1987-1992); the first efficient hydrogen generating solar cells (1980) and electrical power producing photoelectrochemical cells (1978); the first paper on the lithium-thionyl chloride battery used in pacemakers and personal computers, on which 1800 papers were subsequently published by others and which is manufactured worldwide (1972); the introduction of electrochemistry in oxyhalides (1966); and inorganic liquid lasers (1964).

Dr. Heller received the 1978 Battery Research Award of The Electrochemical Society; was named Guest Professor at the Collège de France in 1982; received the David C. Grahame Physical Electrochemistry Award of The Electrochemical Society in 1987; was elected to the U.S. National Academy of Engineering in 1987; received the Vittorio De Nora Gold Medal of The Electrochemical Society in 1988 and was awarded the title of Doctor Honoris Causa by Uppsala University in Sweden in 1991. His distinguished lectureships include the Regents Lectures at UCLA (1984), The Raymond and Beverly Sackler Lectures at Tel Aviv University (1987); The Berkeley Lectures (1991); 200th Anniversary Faraday Lectures of The Electrochemical Society (1991); and The Weizmann Institute's Gerhard M. J. Schmidt Memorial Lecture (1992).

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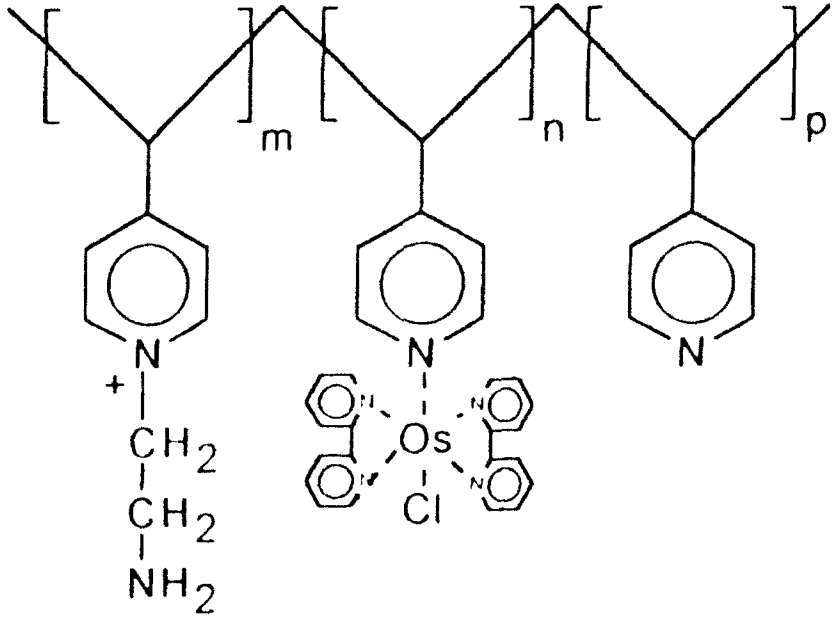
"Amperometric Biosensors Based on 3-
Dimensional Hydrogel-Forming Epoxy Networks"
Figure 1.



$n = 1, m \approx 4, p \approx 1.2$

Adam Heller, Ruben Maidan and DanLi Wang
"Amperometric Biosensors Based on 3-Dimensional Hydrogel-Forming Epoxy Networks"
Figure 2.